ACTIVITIES OF RESPIRATORY ENZYMES DURING THE METAMORPHOSIS OF THE MEDITERRANEAN FLOUR MOTH, EPHESTIA KÜHNIELLA ZELLER[1] (LEPIDOPTERA: PYRALIDAE)

WILLIAM DIAMANTIS[2]
DEPARTMENT OF BIOLOGY, FORDHAM UNIVERSITY
RECEIVED FOR PUBLICATION OCT. 15, 1961

ABSTRACT

The occurrence of a U-shaped respiratory curve during the metamorphosis of holometabolous insects is well established. The roles of dehydrogenase enzymes and cytochrome oxidase in respiratory metabolism have been studied in some insects and these investigations indicate variations in the activities of dehydrogenase enzymes in different insects. In this study the activities of the dehydrogenase enzymes were determined during the metamorphosis of a lepidopteran, *Ephestia kühniella* Zell., using the Thunberg technique. Cytochrome oxidase was determined spectrophotometrically. Six dehydrogenase enzymes exhibited U-shaped activity curves: alpha-glycerophosphate I and II (GPD I and II), malic, isocitric and succinic dehydrogenases and the malic enzyme. The least active of this group was succinic dehydrogenase, adding support to previous work indicating that this enzyme may be a rate-limiting factor in determining the U-shaped respiratory curve. The most active of this group was malic dehydrogenase. Five dehydrogenase enzymes did not exhibit U-shaped activities: lactic I and II, glucose, glutamic and alcohol. The activities of lactic I and II dehydrogenases were low or negligible. The possible significance of this low activity with regards to (a) lactate accumulation and (b) relationship to GPD activity in insects are discussed. The activity of cytochrome oxidase was also found to be U-shaped during metamorphosis.

The occurrence of a U-shaped respiratory curve during the metamorphosis of holometabolous insects was first described by Krogh (1914) for the mealworm, *Tenebrio molitor,* and was confirmed by many investigators with various species of insects (Bodine and Orr 1925, Clare 1925, Fink 1925, Taylor 1927,

[1] A part of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology at Fordham University. The author is greatly indebted to Dr. Daniel Ludwig and expresses his gratitude for the suggestion of this problem and for its direction.

[2] Present address: Wallace Laboratories, Cranbury, New Jersey.

Ludwig 1931, Dobzhansky and Poulson 1935, Wolsky 1938, Sacktor 1951, Ito 1954, Cotty 1956, Ludwig and Barsa 1956a). Comparisons of the oxygen consumption of various insects show considerable variations and Taylor (1927) concluded that there are strong indications of specific differences.

The causative factors responsible for the U-shaped respiratory curve are not definitely understood. The possible role of cytochrome oxidase as a rate-limiting factor in respiratory metabolism has been considered. This enzyme showed a U-shaped activity curve during the metamorphosis of several insects but this type of activity was not universal. A correlation between succinic dehydrogenase activity and oxygen uptake was shown for *Drosophila melanogaster* (Wolsky 1941), *Popillia japonica* (Ludwig and Barsa 1955), *T. molitor* (Ludwig and Barsa 1958) and *Bombyx mori* (Ito 1955). Agrell (1949) showed that total dehydrogenase activity and the activities of malic, succinic, citric and glutamic dehydrogenases each followed U-shaped curves during the metamorphosis of the blow fly, *Calliphora erythrocephala*. However, with the mealworm, Ludwig and Barsa (1958) found malic and succinic dehydrogenases and the malic enzyme activities to be U-shaped during metamorphosis whereas with the house fly, these same investigators (1959) found that in addition, alcohol and alpha-glycerophosphate dehydrogenases also followed U-shaped activity curves. Since these studies indicate that the activities of various dehydrogenases vary in different insects and since no study has been made of the various respiratory enzymes of a lepidopteran, except for the succinoxidase system of the silkworm (Ito 1955), this investigation of cytochrome oxidase and the dehydrogenase activities during the metamorphosis of the Mediterranean flour moth, *Ephestia kühniella* was undertaken.

MATERIAL AND METHODS

Cultures of *E. kühniella* (white eye mutant) were maintained at room temperature (approximately 25°C.) in covered culture dishes containing corn meal. Larvae collected from these cultures were placed at 30°C. in culture dishes with corn meal and examined daily for newly molted pupae. Pupae of known ages, within 24 hours, were thus obtained. Larvae, prepupae and adults used in these determinations were obtained from cultures

kept at 30°C. Stock cultures were kept at room temperature since the higher temperature rendered the males sterile.

The activities of alcohol, glucose, *l*-glutamic, alpha-glycerophosphate, isocitric, lactic, malic, succinic dehydrogenases and the malic enzyme were determined in larvae, prepupae, pupae for each day of the pupal stage, and in newly emerged adults by the Thunberg technique as given by Umbreit, Burris and Stauffer (1957, p. 130) and as modified by Ludwig and Barsa (1958). Insects were homogenized for one minute in 0.30 M phosphate buffer, except for isocitric dehydrogenase, where veronal buffer was used since the phosphate ion interferes with the activity of this enzyme. In all cases the buffers were adjusted to a pH of 7.4. A three per cent homogenate was prepared and incubated at 30°C. for 30 minutes to oxidize the endogenous substrate. For each determination, 0.5 ml. of homogenate was required, and when DPN or TPN was needed the homogenate was incubated with 0.25 ml. of 0.2 per cent DPN or 0.25 ml. of 0.1 per cent TPN. The homogenate or homogenate-coenzyme mixture was pipetted into the side-arm cap of the Thunberg tube. In the body of the tube were placed 0.5 ml. of 1 : 10,000 methylene blue, 0.5 ml. of appropriate substrate (0.04 M), and sufficient buffer to bring the final volume to 3 ml. A control tube containing all the components except the substrate was prepared for each individual determination, and each control tube contained 0.5 ml. of the same homogenate mixture as was used for the determination. In determining malic dehydrogenase activity, 0.25 ml. of 0.24 M KCN was added to each tube to prevent inhibition by the oxaloacetate formed (Green 1936). In measuring the activity of succinic dehydrogenase, 0.25 ml. of a mixture of 0.005 M $CaCl_2$ and 0.005 M $AlCl_3$ was added to each tube for complete activation of this enzyme (Potter and Schneider 1942). TPN was used in the studies of isocitric dehydrogenase and the malic enzyme. In the former determinations, 0.25 ml. of 6×10^{-3} M $MnCl_2$ was added since the system does not react unless the Mn^{++} ion is present (Adler, Euler, Günther and Plass 1939). For the malic enzyme determination, 0.25 ml. of 0.033 M $MgSO_4$ was added to insure activation of the enzyme (Faulkner 1956). In each case the supplementary solutions were added before the final dilution of the homogenate. The tubes, after preparation, were evacuated by vacuum pump for 5 minutes, tapping the

tube to remove dissolved gases. When evacuation was complete, the homogenate was mixed with the other components of the tube, thus bringing the final concentration of the homogenate to 0.5 per cent. The tubes were then placed in a constant temperature bath at 30°C., and the time in minutes required for 90 per cent reduction of methylene blue was determined by visually matching the color with that of the standard tube. This standard contained all components of the other tubes, the homogenate having been previously inactivated by boiling and the methylene blue diluted to 1/10 the usual concentration. Activities of dehydrogenase enzymes are expressed as 1/time in minutes for 90 per cent decoloration of methylene blue. These activities were determined for each of the enzymes as follows:

Activity E (experimental tube) – Activity C (control tube) = Activity of dehydrogenase enzyme

The activity of cytochrome oxidase was determined on the same stages. In each case the enzyme activity was measured on tissue homogenates in a final concentration of 1:10,000. The insects were homogenized in 0.03 M phosphate buffer adjusted to a pH of 7.4. Cytochrome oxidase was measured spectrophotometrically by the method of Cooperstein and Lazarow (1951). Its activity is expressed as $\Delta \log [CyFe^{++}]/min$.

Observations

The changes in activities of various dehydrogenase enzymes during the metamorphosis of the Mediterranean flour moth are given in Table 1. Each value represents a minimum of six determinations, each requiring 3 to 7 insects.

Six dehydrogenases were U-shaped in activity during metamorphosis. They are alpha-glycerophosphate I and II (GPD I and II), malic, isocitric and succinic dehydrogenases and the malic enzyme. In all cases the activity in the newly emerged adult exceeded that of the larva, the greatest difference being found for malic dehydrogenase and the least being for alpha-glycerophosphate I dehydrogenase and the malic enzyme. In addition the low point of activity always occurred during the early pupal stage. Greatest activity was shown by malic dehydrogenase; moderate activity by alpha-glycerophosphate I (requires DPN) and isocitric dehydrogenases and the malic enzyme, and the lowest by succinic dehydrogenase. Alcohol dehydrogen-

Stage	DEHYDROGENASE										
	coenzyme: DPN						coenzyme: TPN		no coenzyme required		
	Alcohol	Glucose	GPD I	Glutamic	Lactic I	Malic	Iso-Citric	Malic enzyme	GPD II	Lactic II	Succinic
Larva	0.008	0.001	0.072	0.012	0.034	0.215	0.064	0.040	0.011	0.005	0.011
Prepupa	0.038	0.001	0.030	0.009	0.009	0.122	0.062	0.014	0.012	0.004	0.002
Pupa, 1 day	0.032	0.006	0.024	0.002	0.009	0.075	0.037	0.020	0.003	0.001	0.002
Pupa, 2 day	0.032	0.004	0.022	0.001	0.008	0.080	0.032	0.016	0.003	0.001	0.002
Pupa, 3 day	0.025	0.004	0.020	0.002	0.007	0.067	0.015	0.018	0.002	0.001	0.001
Pupa, 4 day	0.032	0.002	0.020	0.003	0.007	0.074	0.036	0.021	0.001	0.002	0.001
Pupa, 5 day	0.042	0.004	0.023	0.003	0.009	0.072	0.035	0.024	0.005	0.001	0.003
Pupa, 6 day	0.042	0.002	0.023	0.002	0.010	0.158	0.055	0.030	0.005	0.001	0.009
Pupa, 7 day	0.026	0.002	0.032	0.003	0.008	0.247	0.052	0.022	0.009	0.001	0.010
Pupa, 8 day	0.028	0.003	0.041	0.003	0.007	0.439	0.053	0.023	0.025	0.002	0.021
Adult, newly emerged	0.074	0.006	0.081	0.005	0.007	0.991	0.084	0.049	0.033	0.002	0.035

TABLE 1.

Dehydrogenase activity during the metamorphosis of the Mediterranean flour moth, *Ephestia kühniella*. Activity is expressed as 1/time in minutes for 90 per cent decoloration of methylene blue. The figures listed were obtained by subtracting the value of the control tube from that of the experimental tube. Readings made at 30° C. (GPD is alpha-glycerophosphate dehydrogenase).

ase showed an irregular course of activity, being low in the larva, moderately high in the prepupa and pupal period and reaching a peak in the adult. Lactic I dehydrogenase (requires DPN) exhibited low activity while lactic II dehydrogenase activity was found in the larva and prepupa only. Glucose dehydrogenase activity was absent in the larva and prepupa and low during the rest of metamorphosis. Glutamic dehydrogenase, on the other hand, showed activity in the larva and prepupa, but was absent or showed only slight activity in the pupal and adult stages.

Cytochrome oxidase was also U-shaped in activity during metamorphosis (Table 2). Activity in the larva was high at

Stage	Enzyme Activity Δlog [$CyFe^{++}$]/min.		
	Minimum	Maximum	Average
Larva	0.033	0.085	0.065
Prepupa	0.022	0.067	0.036
Pupa, 1 day	0.009	0.022	0.015
Pupa, 2 day	0.005	0.014	0.010
Pupa, 3 day	0.009	0.018	0.012
Pupa, 4 day	0.006	0.013	0.009
Pupa, 5 day	0.015	0.038	0.025
Pupa, 6 day	0.044	0.080	0.065
Pupa, 7 day	0.042	0.093	0.068
Pupa, 8 day	0.092	0.127	0.110
Adult, newly emerged	0.083	0.178	0.134

TABLE 2.

Cytochrome oxidase activity during the metamorphosis of *Ephestia kühniella*. Enzyme activity is expressed as Δlog [$CyFe^{++}$]/min. Homogenate concentration is 1:10,000.

0.065, decreasing to 0.009 in the 4-day pupa, and increasing rapidly during the remainder of the pupal stage reaching 0.134 in the adult.

Discussion

These results and those of Agrell (1949) with the blow fly, *C. erythrocephala,* Ludwig and Barsa (1958, 1959) with the mealworm and the house fly, show that malic and succinic dehydrogenases and the malic enzyme consistently show U-shaped activities during metamorphosis in all insects in which they have been studied. They are involved in the oxidation-reduction

reactions of the tricarboxylic acid cycle. Malic dehydrogenase accounted for a very large portion of total activity, being the most active enzyme during the metamorphosis of the flour moth. In the mealworm and the house fly (Ludwig and Barsa 1958, 1959) it showed high activity but was not always the most active enzyme throughout metamorphosis, its activity being exceeded in some stages by that of isocitric dehydrogenase. Malic enzyme was less active in the flour moth than in either the mealworm or house fly. Both malic dehydrogenase and the malic enzyme catalyze the oxidation of *l*-malate, the end product with malic dehydrogenase being oxaloacetate; whereas with the malic enzyme, they are pyruvate and CO_2. In this latter reaction there is no evidence that oxaloacetate is an intermediate (Veiga Salles and Ochoa 1950). Faulkner (1956) has shown in insect blood that the oxidation of malate to pyruvate by the malic enzyme can be reversed.

The low activity of lactic I and II dehydrogenases is in agreement with the results of Ludwig and Barsa (1959) but not with Agrell (1949) who indicated that lactic dehydrogenase activity was moderately high during the metamorphosis of the blow fly. The low activity for total lactic dehydrogenase combined with the high rates of malic dehydrogenase and the malic enzyme lend support to the hypothesis of Ludwig and Barsa (1958) that in insects lactate does not accumulate, but rather that pyruvate is reduced to malate which is then oxidized to oxaloacetate.

The observation that the activity of alpha-glycerophosphate dehydrogenase I was greater than that of alpha-glycerophosphate dehydrogenase II in the flour moth agrees with that of Zebe and McShan (1957) with various species of insects. These authors suggest that the oxidation-reduction of alpha-glycerophosphate, although relatively unimportant in vertebrates, must be very important in insects as indicated by the higher activity of alpha-glycerophosphate I dehydrogenase in insects. In addition, the low rate of lactic and high rate of alpha-glycerophosphate I dehydrogenases found in the flour moth lends support to the work of Zebe and McShan (1957) who showed that the activities of these two enzymes were related. They found that in insect flight muscles, the activity of lactic dehydrogenase was low and alpha-glycerophosphate dehydrogenase was high, whereas in some special cases of leg muscle the related activities

were exactly reversed. They concluded that in different muscles, at least to a degree, one enzyme might take the place of the other.

Isocitric dehydrogenase activity in the flour moth was not only considerably lower than that found in both the mealworm and house fly (Ludwig and Barsa 1958, 1959), but the course of the activity differed. Whereas in the flour moth and the blow fly (Agrell 1949) its activity was U-shaped, with the mealworm and house fly it decreased steadily throughout metamorphosis. This enzyme in the presence of TPN and Mn^{++} catalyzes the oxidation of isocitrate through oxalosuccinate to alpha-ketoglutarate. The activity of succinic dehydrogenase was uniformly low in all insects studied; and in fact was the lowest of all enzymes whose activity is U-shaped. This observation suggests that it could be the rate-limiting factor in determining the U-shaped respiratory curve.

The observation that cytochrome oxidase activity follows a U-shaped course during metamorphosis of the flour moth is in agreement with that of Wolsky (1938), Williams (1950), Sacktor (1951) and Ludwig (1953) with the fruit fly, *D. melanogaster,* the moth, *Platysamia cecropia,* the house fly, *M. domestica,* and the Japanese beetle, *P. japonica,* respectively. However, it is not in accord with the findings of Ito (1955) with the silk worm, or of Ludwig and Barsa (1956b) with the mealworm. Ito (1955) concluded that, whereas cytochrome oxidase activity shows a curve similar to that of oxygen uptake after the middle of the pupal period, it does not parallel oxygen uptake during the early part of this stage. Thus, while cytochrome oxidase may be a terminal oxidase, it is not the rate-limiting enzyme throughout the entire course of metamorphosis in all insects studied.

Summary

A study was made of the activities of dehydrogenase enzymes during the metamorphosis of the Mediterranean flour moth using the Thunberg technique. The activity of cytochrome oxidase during this same period was determined spectrophotometrically.

Six dehydrogenase enzymes exhibited U-shaped activity curves. They are alpha-glycerophosphate I and II, malic, isocitric and succinic dehydrogenases and the malic enzyme. The most active enzyme of this group was malic dehydrogenase and the least active was succinic dehydrogenase. Moderate activity was shown

by alpha-glycerophosphate I and isocitric dehydrogenases and the malic enzyme. Alpha-glycerophosphate II dehydrogenase showed low activity.

The activities of lactic I and II, glucose and glutamic dehydrogenases were low or negligible, whereas that of alcohol dehydrogenase was irregular.

The low activity rate for total lactic dehydrogenase combined with the high rate of malic dehydrogenase and the malic enzyme lends support to the hypothesis that lactate does not accumulate in insects, but rather that pyruvate is reduced to malate which is then oxidized to oxaloacetate. Additionally, the low rate of lactic and high rate of alpha-glycerophosphate I dehydrogenases lend support to work indicating that the activities of these two enzymes are related in insect muscles and this relationship may be reversed in certain muscles.

With the exception of alpha-glycerophosphate I and II, the results indicate that the dehydrogenases showing U-shaped activity curves are involved in oxidation-reduction reactions of the tricarboxylic acid cycle. The rate of succinic dehydrogenase being the lowest of those exhibiting U-shaped activity curves, adds support to previous work indicating that this enzyme may be a rate-limiting factor in determining the U-shaped respiratory curve.

The activity of cytochrome oxidase was also found to be U-shaped during metamorphosis.

Literature Cited

ADLER, E., H. v. EULER, G. GÜNTHER AND M. PLASS, 1939. Isocitric dehydrogenase and glutamic acid synthesis in animal tissue. Biochem. J., 33: 1028–1045.

AGRELL, I. P. S., 1949. Localization of some hydrogen-activating enzymes in insects during metamorphosis. Nature, 164: 1039–1040.

BODINE, J. H. AND P. R. ORR, 1925. Respiratory metabolism. Biol. Bull., 48: 1–14.

CLARE, M. R., 1925. A study of oxygen metabolism in *Drosophila melanogaster*. Biol. Bull., 49: 440–460.

COOPERSTEIN, S. J. AND A. LAZAROW, 1951. A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem., 189: 665–670.

COTTY, V. F., 1956. Respiratory metabolism of prepupae and pupae of the house fly, *Musca domestica* L., and of their homogenates. Contrib. Boyce Thompson Inst., 18: 253–262.

DOBZHANSKY, T. AND D. F. POULSON, 1935. Oxygen consumption of *Drosophila* pupae. II. *Drosophila pseudobscura*. Z. vergl. Physiol., **22**: 473–478.

FAULKNER, P., 1956. The "malic" emzyme in insect blood. Biochem. J., **64**: 430–436.

FINK, D. E., 1925. Metabolism during embryonic and metamorphic development of insects. J. Gen. Physiol., **7**: 527–543.

GREEN, D. E., 1936. The malic dehydrogenase of animal tissue. Biochem. J., **30**: 2095–2110.

ITO, T., 1954. The physiology in the metamorphosis of *Bombyx mori*. I. Respiration. Bull. Sericult, Exp. Sta. (Tokyo), **14**: 263–278.

———, 1955. The physiology in the metamorphosis of *Bombyx mori*. II. Succinoxidase system in the pupal stage. Annot. Zool. Japonse, **28**: 1–7.

KROGH, A., 1914. On the rate of development and CO_2 production of chrysalides of *Tenebrio molitor* at different temperatures. Z. allg. Physiol., **16**: 178–190.

LUDWIG, D., 1931. Studies on the metamorphosis of the Japanese beetle (*Popillia japonica* Newman). I. Weight and metabolism changes. J. Exp. Zool., **60**: 309–323.

————, 1953. Cytochrome oxidase activity during diapause and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). J. Gen. Physiol., **36**: 751–757.

———— AND M. C. BARSA, 1955. The activity of succinic dehydrogenase during diapause and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). J. N. Y. Ent. Soc. **63**: 161–165.

————————, 1956 a. Oxygen consumption of whole insects and insect homogenates. Biol. Bull., **110**: 77–82.

————————, 1956 b. Variations in the activity of the succinoxidase system during the development of the mealworm, *Tenebrio molitor* (Coleoptera, Tenebrionidae). Ann. Ent. Soc. Amer., **49**: 103–104.

————————, 1958. Activity of dehydrogenase enzymes during the metamorphosis of the mealworm, *Tenebrio molitor* Linnaeus. Ibid, **51**: 311–314.

————————, 1959. Activities of respiratory enzymes during the metamorphosis of the housefly, *Musca domestica* Linnaeus. J. N. Y. Ent. Soc., **67**: 151–156.

POTTER, V. R. AND W. C. SCHNEIDER, 1942. Studies on the mechanism of hydrogen transport in animal tissue. V. Dilution effects in the succinoxidase system. J. Biol. Chem., **142**: 543–555.

SACKTOR, B., 1951. Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, *Musca domestica* L. Biol. Bull., **100**: 229–243.

TAYLOR, I. R., 1927. Oxygen consumption of individual pupae during metamorphosis. J. Morph., **44**: 313–339.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER, 1957. Manometric

techniques. A manual describing methods applicable to the study of tissue metabolism. Minneapolis.

VEIGA SALLES, J. B. AND S. OCHOA, 1950. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. II. Further study of the properties of "malic" enzyme of pigeon liver. J. Biol. Chem., 187: 849–861.

WILLIAMS, C. M., 1950. A hormonal-enzymatic mechanism for control of pupal diapause in the *Cecropia* silkworm. Abstract of Communication to the XVIII Internat. Physiol. Cong. (Copenhagen), 517–518.

WOLSKY, A., 1938. The effect of carbon monoxide on oxygen consumption of *Drosophila melanogaster* pupae. J. Exp. Biol., 15: 225–234.

————, 1941. Quantitative changes in the substrate-dehydrogenase system of *Drosophila* pupae during metamorphosis. Science, 94: 48–39.

ZEBE, E. C. AND W. H. MCSHAN, 1957. Lactic and alpha-glycerophosphate dehydrogenase in insects. J. Gen. Physiol., 40: 779–790.